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Additional inventors are being named on the 1 *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

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[Page 1 of 2]

Respectfully submitted,

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RNA EXTRACTION METHOD AND BIOMARKERS FOR ORAL CAVITY AND OROPHARYNGEAL CANCER

BY

David T. W. Wong; Maie A. R. St. John; and Yang Li

Field of the disclosure

[0001] The present disclosure relates to methods to isolate RNA in saliva, to biomarkers for oral cavity and oropharhyngeal cancers and to method and kit using said biomarkers.

[0002] This invention was made with Government support of grant U01-DE15018 awarded by the NIH. The Government has certain rights on this invention

Background of the disclosure

[0003] Saliva is not a passive "ultrafiltrate" of serum (Rehak *et al.*, 2000), but contains a distinctive composition of enzymes, hormones, antibodies, and other molecules. In the past 10 years, the use of saliva as a diagnostic fluid has been successfully applied in diagnostics and predicting populations at risk for a variety of conditions (Streckfus and Bigler, 2002).

[0004] Diagnostic biomarkers in saliva have been identified for monitoring caries, periodontitis, oral cancer, salivary gland diseases, and systemic disorders, *e.g.*, hepatitis and HIV (Lawrence, 2002). Human genetic alterations are detectable both intracellularly and extracellularly (Sidransky, 1997). Nucleic acids have been identified in most bodily fluids including blood, urine and cerebrospinal fluid, and have been successfully adopted for using as diagnostic biomarkers for diseases (Anker, *et al.*, 1999; Rieger-Christ, *et al.*, 2003; Wong, *et al.*, 2003).

[0005] Recent interest has developed to detect nucleic acid markers in saliva. To date, most of the DNA or RNA in saliva was found to be of viral or bacterial origin

(Stamey, *et al.,* 2003; Mercer *et al.*, 2001). There are a limited number of reports demonstrating tumor cell DNA heterogeneity in saliva of oral cancer patients (Liao, *et al.*, 2000; El-Naggar, *et al.*, 2001). We have not found published evidence of human mRNA detectable in saliva. The potential presence of mRNA in saliva may expand the repertoire of diagnostic analytes for translational and clinical applications.

[0006] There is a need for such analytes or biomarkers especially with reference to cancer related applications.

[0007] Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer in the world, and affects 50,000 Americans annually. Worldwide, cancers of the oral cavity (OC) and oropharynx (OP) represent a great public health problem. Squamous cell carcinoma (SCCA) of the OC accounts for nearly 50% of all newly diagnosed cancers in India and is a leading cause of death in France [1].

[0008] Despite improvements in locoregional control, morbidity and mortality rates have improved little in the past 30 years [2]. Therefore, early detection or prevention of this disease is likely to be most effective. Detecting HNSCC at an early stage is believed to be the most effective means to reduce death and disfigurement from this disease. The absence of definite early warning signs for most head and neck cancers suggests that sensitive and specific biomarkers are likely to be important in screening high risk patients. A number of molecular markers have been used to detect these tumors with varying degrees of specificity and sensitivity. DNA markers include TP53, microsatellite instability (MSI), and the presence of the human papilloma virus (HPV) and the Epstein-Barr virus (EBV) genomic sequences [3]. None of these markers has been shown to universally identify OSCC.

Summary of the disclosure

[0009] According to a first aspect, a method to isolate mRNA from saliva is disclosed, comprising: providing a cell-free saliva supernatant; and isolating mRNA from the cell free saliva supernatant.

[0010] In particular, providing a cell-free saliva supernatant preferably comprises: providing unstimulated saliva; and deriving the cell-free saliva supernatant from the unstimulated saliva.

[0011] According to a second aspect, a method to perform transcriptome analysis of saliva is disclosed, comprising providing cell-free saliva supernatant; and detecting a transcriptome pattern in the saliva supernatant.

[0012] In particular, detecting transcriptome pattern in the saliva supernatant is preferably performed by microarray assay, most preferably by high-density oligonucleotide microarray assay. Detecting transcriptome pattern in the saliva supernatant can also performed by quantitative PCR analysis or RT-PCR analysis.

[0013] According to a third aspect, a method to detect genetic alterations in an organ by analyzing a bodily fluid draining from the organ, is disclosed. The bodily fluid is in particular saliva and method comprises: providing cell-free saliva supernatant; detecting a transcriptome pattern in the saliva supernatant; and comparing the transcriptome pattern with a predetermined pattern, the predetermined pattern being indicative of a common pattern transcriptome of normal cell-free saliva.

[0014] According to a fourth aspect, a method to detect genetic alteration of a gene in an organ by analyzing a bodily fluid draining from the organ, is disclosed. The bodily fluid is in particular saliva and the method comprises: providing cell-free saliva supernatant; detecting an mRNA profile of the gene in the saliva supernatant; and comparing the mRNA profile of the gene with a predetermined mRNA profile of the gene, the predetermined mRNA profile of the gene being indicative of the mRNA profile of the gene in normal cell-free saliva.

[0015] According to a fifth aspect, a method to diagnose an oral or systemic disease in a subject, is disclosed the method comprising: providing cell-free saliva supernatant of the subject; detecting in the cell-free saliva supernatant an mRNA profile of a gene associated with the disease; and compare the RNA profile of the gene with a

predetermined mRNA profile of the gene, the predetermined mRNA profile of the gene being indicative of the presence of the disease in the subject.

[0016] In a first embodiment the disease is a cancer of the oral cavity and/or of oropharynx and the gene is selected from the group consisting of the gene coding for IL8, IL1B, DUSP1, HA3, OAZ1, S100P and SAT.

[0017] In a second embodiment the disease is a cancer of the oral cavity and/or a oropharynx and the gene is the gene coding for IL8.

[0018] Diseases that can be diagnosed include oropharyngeal Squamous cell carcinoma, head and neck squamous cell carcinoma, breast cancer and diabetes.

[0019] According to a sixth aspect a method to diagnose an oral or systemic disease in a subject is disclosed, the method comprising: providing cell-free saliva supernatant of the subject; detecting in the cell-free saliva supernatant a transcriptome pattern associated with the disease; and comparing the transcriptome pattern with a predetermined pattern, wherein the recognition in the transcriptome pattern of characteristic of the predetermined pattern is diagnostic for the disease in the subject.

[0020] In an embodiment the disease is a cancer of the oral cavity and/or of oropharynx, and transcriptome include transcript is selected from the group consisting of transcripts for IL8, IL1B, DUSP1, HA3, OAZ1, S100P and SAT.

[0021] Diseases that can be diagnosed include oropharyngeal Squamous cell carcinoma, head and neck squamous cell carcinoma, breast cancer and diabetes.

[0022] According to a seventh aspect, a method for diagnosing a cancer in a subject is disclosed, the method comprising: providing a bodily fluid of the subject; detecting in the bodily fluid a profile of a biomarker, the biomarker selected from the group consisting of IL6, IL8 IL1B, DUSP1, HA3, OAZ1, S100P and SAT; and comparing the profile of the biomarker with a predetermined profile of the biomarker, wherein the

recognition in the profile of the biomarker of characteristics of the predetermined profile of the biomarker being diagnostic for the cancer.

[0023] In a first embodiment, the biomarker is selected from the group consisting of IL8, IL1B, DUSP1, HA3, OAZ1, S100P and SAT, the bodily fluid is saliva and detecting a profile of a biomarker is performed by detecting the mRNA profile of the biomarker.

[0024] In a second embodiment, the biomarker is IL6, the bodily fluid is blood serum and detecting a profile of a biomarker is performed by detecting the mRNA profile of the biomarker.

[0025] In a third embodiment, the biomarker is IL6, the bodily fluid is blood serum and detecting a profile of a biomarker is performed by detecting the protein profile of the biomarker.

[0026] Diseases that can be diagnosed include oropharyngeal Squamous cell carcinoma, head and neck squamous cell carcinoma, breast cancer and diabetes.

[0027] According to an eighth aspect a kit for the diagnosis of an oral and/or systemic disease is disclosed, the kit comprising: an identifier of at least one biomarker in a bodily fluid, the biomarker selected from the group consisting of IL8, IL1B, DUSP1, HA3, OAZ1, S100P and SAT; and a detector for the identifier.

[0028] The identifier and the detector are to be used in detecting the bodily fluid profile of the biomarker according to the diagnostic methods herein disclosed. In particular, the identifier is associated to the biomarker in the bodily fluid, and the detector is used to detect the identifier, the identifier and the detector thereby enables the detection of the bodily fluid profile of the biomarker.

[0029] Diseases that can be diagnosed include oropharyngeal Squamous cell carcinoma, head and neck squamous cell carcinoma, breast cancer, HIV and diabetes.

[0030] According to a ninth aspect a method is disclosed, comprising: using salivary mRNAs as biomarkers for oral and/or systemic diseases.

[0031] In a preferred embodiment the mRNA codify for IL8, IL1B, DUSP1, HA3, OAZ1, S100P and SAT.

[0032] Diseases that can be diagnosed include oropharyngeal Squamous cell carcinoma, head and neck squamous cell carcinoma, breast cancer and diabetes.

[0033] The methods and kits of the disclosure will be exemplified with the aid of the enclosed figures.

Description of the figures

[0034] **Figure 1:** Detection of gene specific RNA in cell-free saliva using RT-PCR. (A) RNA stability in saliva was tested by RT-PCR typing for ACTB after storage for 1, 3, and 6 months (lanes 2, 3, 4 respectively). Lane 1, molecular weight marker (100bp ladder); Lane 5, negative control (omitting templates). (B) GAPDH ***(B1)***, RPS9 ***(B2)*** and ACTB ***(B3)*** were detected consistently in all 10 cases. Lanes 1, 2 and 3 are saliva RNA, positive control (human total RNA, BD Biosciences Clontech, Palo Alto, CA, USA) and negative controls (omitting templates), respectively.

[0035] **Figure 2:** Amplification of RNA from cell-free saliva for microarray study. (A) Monitoring of RNA amplification by agarose gel electrophoresis. Lanes 1 to 5 are 1kb DNA ladder, 5µl saliva after RNA isolation (undetectable), 1µl two round amplified cRNA (range from 200 bp to ~4kb), ***1µl*** cRNA after fragmentation (around 100bp) and Ambion RNA Century Marker, respectively. (B) ACTB can be detected in every main step during salivary RNA amplification. The agarose gel shows expected single band (153bp) of PCR product. Lane 1 to 8 are 100bp DNA ladder, total RNA isolated from cell-free saliva, 1st round cDNA, 1st round cRNA after RT, 2nd round cDNA, 2nd round cRNA after RT, positive control (human total RNA, BD Biosciences Clontech, Palo Alto, CA, USA) and negative control (omitting templates), respectively. (C) Target cRNA analyzed

by Agilent 2100 bioanalyzer before hybridization on microarray. Only one single peak in a narrow range (50-200bp) was detected demonstrating proper fragmentation.

[0036] **Figure 3:** IL-6 and IL-8 mrna transcripts are present in the fluid phase of saliva. Using reverse transcriptase-polymerase chain reaction (RT-PCR), we were able to demonstrate that IL-6 and IL-8 mRNA transcripts are present in the fluid phase in saliva. The PCR products had the sizes (95 bp and 88 bp, respectively) that were expected from the selected primers.

[0037] **Figure 4:** Optimization of centrifugation speed for saliva. In order to ensure that the RNA and protein analyzed were from the fluid phase of saliva only (and to ensure the lack of contamination by intracellular components), we optimized the centrifugation speed for the saliva and serum samples. We utilized genomic DNA as a marker of cell lysis and spillage of intracellular components. We performed PCR for the housekeeping gene β-actin on whole saliva and serum samples, and samples that had been centrifuged at various speeds. We were able to determine the optimal centrifugation speeds at which there was no spillage of intracellular contents. Saliva was then centrifuged at 2600xg; blood was centrifuged at 1000xg.

[0038] **Figure 5:** IL-8 levels are significantly higher in the saliva of OSCC patients. Concentrations of IL-8 in saliva from patients with OSCC and normal subjects. The levels of IL-8 in saliva from age- and gender-matched patients with OSCC and normal subjects were measured by qRT-PCR (mRNA copy number, *A*), and ELISA (pg/ml, *B*). The mean concentration of IL-8 detected in replicate samples in saliva in patients with cancer, and normal subjects is shown. *Solid bars*, mean concentration (± SEM) of samples for each group. A significant difference by *t* test between affected and normal subjects at $P < 0.05$.

[0039] **Figure 6:** IL-6 levels are significantly higher in the serum of OSCC patients. Concentrations of IL-6 in serum from patients with OSCC and normal subjects. The levels of IL-6 in serum from age- and gender-matched patients with OSCC and normal subjects were measured by qRT-PCR (mRNA copy number, *A*), and ELISA (pg/ml, *B*).

The mean concentration of IL-6 detected in replicate samples in serum in patients with cancer, and normal subjects is shown. *Solid bars*, mean concentration (± SEM) of samples for each group. A significant difference by *t* test between affected and normal subjects at $P < 0.05$.

[0040] **Figure 7:** ROC (Receiver Operating Characteristic) Curves (A) IL-8 in Saliva; (B) IL-6 in Serum; (C) IL-8 in Saliva and IL-6 in Serum. ROC (Receiver Operating Characteristic) curves, plots of sensitivities versus 1-specificities, were generated for each of the potential biomarkers. The areas under the ROC curves were calculated, as measures of the utility of each biomarker for detecting OSCC (Table 3(1)). For IL-8 in saliva, a threshold value of 600 pg/mL yields a sensitivity of 86% and a specificity of 97%. For IL-6 in serum, a threshold value of greater than 0 pg/mL yields a sensitivity of 57% and a specificity of 100%. For a combination of IL-8 protein in saliva and IL-6 protein in serum, a threshold value of > 600 pg/mL IL-8 in saliva and > 0 pg/ml IL-6 in serum yields a sensitivity of 99% and a specificity of 90%.

Detailed description of the disclosure

[0041] The methods and kits of this disclosure have been derived as a result of a study which is disclosed herein below.

[0042] The inventors have been actively involved in the application of patient-based genome-wide technologies to identify molecular biomarkers from saliva. A series of emerging technologies to detect diagnostic analytes in saliva has been applied.

[0043] The inventors have identified salivary human interleukin 8 mRNA and protein to be diagnostic of patients with oral cavity and pharyngeal cancer.

[0044] Based on this finding, the inventors hypothesized that there are constituent human mRNAs in saliva. The purpose of the present disclosure was to determine the transcriptome profiles in cell-free saliva obtained from normal subjects. High-density oligonucleotide microarrays were used for the global transcriptome profiling. The salivary transcriptome patterns were used to generate a reference database for salivary transcriptome diagnostics applications.

[0045] Saliva, like other bodily fluids, has been used to monitor human health and disease. This disclosure tests the hypothesis that informative human mRNA exists in cell-free saliva. If present, salivary mRNA may provide potential biomarkers to identify populations and patients at high risk for oral and systemic diseases. *Unstimulated saliva* was collected from ten normal subjects. RNA was isolated from the cell-free saliva supernatant and linearly amplified. High-density oligonucleotide microarrays were used to profile salivary mRNA. The results demonstrated that there are thousands of human mRNAs in cell-free saliva.

[0046] Quantitative PCR (Q-PCR) analysis confirmed the present of mRNA identified by our microarray study. A reference database was generated based on the mRNA profiles in normal saliva. The present disclosure proposes a novel clinical approach to

salivary diagnostics, Salivary Transcriptome Diagnostics (STD), for potential applications in disease diagnostics as well as normal health surveillance.

[0047] Saliva meets the demands of an inexpensive, non-invasive and accessible bodily fluid to act as an ideal diagnostic medium. Specific and informative biomarkers in saliva are greatly needed to serve for diagnosing disease and monitoring human health (Bonassi *et al.*, 2001; Streckfus and Bigler, 2002; Sidransky, 2002). Knowing the constituents in saliva is essential for using this medium to identify potential biomarkers for disease diagnostics (Pusch *et al.*, 2003). One criticism has been the idea that informative molecules are generally present in low amounts in saliva.

[0048] However, with new amplification techniques and highly sensitive assays, this may no longer be a limitation (Xiang *et al.*, 2003). In accordance with the present disclosure, the human RNA was successfully isolated from ***unstimulated*** cell-free saliva supernatant. The quality of salivary mRNA was proved to be sufficient for use in RT-PCR, Q-PCR and microarray experiments. Distinct difference exists between saliva and other bodily fluids (*e.g.,* blood) in that saliva naturally contains microorganisms (Sakki and Knuuttila, 1996). In addition, some extraneous substances (*e.g.,* food debris) make the composition of saliva more complex. Therefore, it is simpler and more accurate to use the fluid/supernatant phase of saliva, instead of the whole saliva as medium for detecting biomarkers.

[0049] According to the present disclosure, the conditions for separating the pellet and saliva supernatant were optimized to avoid mechanical rupture of cellular elements which would contribute to the RNA detected in the fluidic cell-free phase. The results of the disclosure demonstrate that it is feasible and efficient to use cell-free saliva for transcriptome analysis. While it is a novel finding that human mRNAs exist in cell-free saliva supernatant, nucleic acids have long been detected in other cell-free bodily fluids and subsequently used for disease diagnostics. For example, specific oncogene, tumor suppressor gene and microsatellite alterations have been identified in patients' serum (Anker *et al.*, 2003). Moreover, tumor mRNAs have been isolated and amplified from

serum of patients with different malignancies (Kopreski, *et al.*, 1999; Fleischhacker, *et al.*, 2001). It has been widely accepted that these genomic messengers detected extracellularly can serve as biomarkers for diseases (Sidransky, 1997).

[0050] In accordance with the present disclosure, human mRNA in ***unstimulated*** saliva is globally profiled. Using microarray technology, the inventors discovered that approximately 3,000 different human mRNAs exist in cell-free saliva of each normal subject. The salivary transcriptome pattern in cell-free saliva from normal populations could potentially serve as a health-monitoring database. It should be noted that we now know the human genome composed of more than 30,000 genes (Venter, *et al.*, 2001) and the probe sets on HG U133A microarray used by the inventors represent only ~19,000 human genes. Additional gene transcripts not detectable by the HG U133A microarray will likely exist in the cell-free saliva. Therefore, it is reasonable to predict that more human mRNAs will be identified in saliva by other advanced methodologies. The identified gene transcripts in the present disclosure, particularly the Normal Salivary Core Transcriptome (NSCT) mRNAs, represent the common transcriptome of normal cell-free saliva.

[0051] According to the present disclosure that human RNA can be isolated, amplified and profiled from cell-free saliva. This advances the concept that saliva has the potential to be a key medium for detecting and monitoring human health and disease. Moreover, the present disclosure provides new insights into previously unnoticed biological processes, such as the release and clearance of RNA in saliva. The origin of human mRNA found in saliva remains to be an important biological question that needs to answer.

[0052] The inventors hypothesize that different, informative and diagnostic transcriptome can be identified in saliva from patients with various disease conditions. Human salivary mRNA can be used as diagnostic biomarkers for oral and systemic diseases that may be manifested in the oral cavity. In particular, salivary mRNA can be

used as diagnostic biomarkers for cancer that may be manifested and/or affect the oral cavity.

[0053] It is possible that saliva-based mRNA assays have the needed specificity and sensitivity for reliable diagnostics. This innovative approach, salivary transcriptome diagnostics (STD), can provide new opportunities for early diagnostics of oral and systemic diseases.

[0054] The inventors have been actively involved in the application of patient-based genome wide technologies to identify molecular biomarkers specific for OSCC. Since morbidity and mortality rates due to oral cavity and oropharyngeal squamous cell carcinoma (OSCC) have improved little in the past 30 years, early detection or prevention of this disease is likely to be most effective. Using laser-capture microdissection and global gene expression profiling using high-density oligonucleotide arrays, we have identified the expression of two cellular genes which are uniquely associated with OSCC: IL-6 and IL-8. [4] These cytokines have also been linked with increased tumor growth and metastasis, and could thus contribute to the pathogenesis of this disease.[5] Their expression is silenced in normal keratinocytes. Others have also detected elevated concentrations of IL-6 and IL-8 in cell-line supernatants, tumor specimens, and the serum of patients with HNSCC. [5]

[0055] Genetic alterations can be successfully identified in bodily fluids draining the organ affected by the tumor.[6] With this in mind, the inventors investigated whether the ability to analyze saliva for potential biomarkers would be feasible in the diagnosis of OSCC. In particular to investigate whether IL-6 and/or IL-8 could serve as informative biomarkers for OSCC in patient saliva and/or serum; to determine if there is a role for saliva as a diagnostic medium for OSCC.

[0056] Specifically, the inventors examined IL-6 and IL-8 at the messenger RNA (mRNA) and the protein levels in both the serum and saliva of OSCC patients and age- and gender-matched controls. Furthermore, the data were subjected to statistical

analysis in order to determine the specificity and sensitivity of these biomarkers for OSCC, as well as their predictive value.

[0057] In the present disclosure, the inventors set out to identify whether two specific cytokines, IL-6 and IL-8, could be measured in the saliva and serum of patients with OSCC, and whether these cytokines could potentially be useful as biomarkers for head and neck cancers. IL-8 was detected at higher concentrations in the saliva of patients with OSCC ($P < 0.01$); and IL-6 was detected at higher concentrations in the serum of patients with OSCC ($P < 0.01$). The inventors confirmed these results at both the mRNA and the protein levels, and the results were concordant. The concentration of IL-8 in saliva and IL-6 in serum did not appear to be associated with gender, age, or alcohol or tobacco use ($P > 0.75$). The inventors subjected the data to statistical analysis, in particular to ROC analysis, and were able to determine the threshold value, sensitivity, and specificity of each biomarker for detecting OSCC (Table 3). Furthermore, the inventors were able to measure mRNA in salivary specimens.

[0058] Numerous studies have shown that genetic alterations can be successfully identified in bodily fluids that drain from the organ affected by the tumor.[6] The ability to analyze saliva would therefore be beneficial in the diagnosis and treatment of OSCC. The use of saliva has been criticized as a diagnostic medium since informative analytes are generally present in lower amounts than in serum. However, with new amplification techniques and highly sensitive assays, this objection is no longer valid. We tested the hypothesis that IL-6 and IL-8 mRNA transcripts are present in the fluid phase in saliva by using RT-PCR. The RT-PCR studies demonstrated that saliva and serum indeed contained mRNA encoding for IL-6 and IL-8 (See example 4).

[0059] The use of the fluid phase of saliva has unique advantages over the use of exfoliated cells. Depending on the location of the tumor, one may not be able to easily access and swab the tumor bed. Although salivary biomarkers could not identify the site from which the tumor originated, they could identify patients at risk. Such a saliva test could be administered by nonspecialists in remote locations as a screening tool to

select patients for referral for careful evaluation of the upper aerodigestive tract. Finding early stage, previously undetected disease may ultimately save lives. Moreover, the use of easily accessible biomarkers may prove highly beneficial in large populations or chemoprevention trials.

[0060] Other studies have supported a role for IL-6 and IL-8 in OSCC [5][11][12] [13]. Preliminary results of an analysis of the effect of surgery or chemo- or radiotherapy upon IL-6 levels indicate that serum cytokine levels decrease in post-treatment patients.[14] Using the Affymetrix 133A high-density oligonucleotide arrays our laboratory has independently profiled the salivary transcriptome of 10 of the 32 OSCC samples and confirmed IL-8 was significantly overexpressed (>2 fold) in all samples examined ($P<0.05$).

[0061] Accordingly IL-6 and IL-8 may therefore play a role in the pathogenicity of OSCC as well as serve as useful biomarkers. Elevation of IL-6 has been shown to promote immune unresponsiveness and induction of wasting, cachexia, and hypercalcemia, all of which are observed in patients with OSCC who have a poor prognosis.[15][16] IL-8 plays an important role in the stimulation of angiogenesis, proliferation, and chemotaxis of granulocytes and macrophages, which are prominent constituents in the stroma of OSCCs.[14][15] In our study, the IL-6 levels in serum, and IL-8 levels in the saliva of OSCC patients were all higher than the determined cutoff value (Figure 7). Variations in biomarker levels in OSCC patients may be attributed to a number of factors, including: submucosal tumor growth, or differences in individual tumor host inflammatory responses. The fact that no healthy control subject had a saliva or serum marker above the reported cutoff is encouraging, indicating the excellent specificity of these tests.

[0062] The inventors are aware of the role and association of IL-6 and IL-8 in various inflammatory conditions in the oral cavity (e.g. periodontal diseases) and serum. While the inventors did not stratify the patient population according to these conditions, the outcome of the pooled analysis was statistically significant with respect to the levels of

these two pro-inflammatory cytokines between OSCC patients and normal controls. This suggests that the OSCC contribution to the elevation of IL-6 and IL-8 in saliva and serum is significantly above the background contribution by the host's potential inflammatory conditions that may affect these two cytokines in saliva and serum.

[0063] The findings of the inventors indicate that IL-8 in saliva, and IL-6 in serum may hold promise as biomarkers for OSCC. According to the National Cancer Institute's "Early Detection Research Network (EDRN)", which defines biomarker validation as a 5 phase process [17], this study represents the completion of the second phase. Phase 3 will be a retrospective longitudinal study including different patient groups (i.e. OSCC of different stages including precancers and controls including other oral diseases).

[0064] A saliva-based test could be a cost-effective adjunct diagnostic tool in the postoperative management of OSCC patients. It could potentially be used for monitoring the efficacy of treatment, or disease recurrence after therapy has concluded. IL-6 and IL-8 may also serve as prognostic indicators to direct the treatment of patients with head and neck cancer. In the future, high-risk patients can be directed to more aggressive or adjuvant treatment regimens.

[0065] The use of these biomarkers may also improve the staging of the tumor. With traditional techniques, the presence of microscopic distant disease is often under recognized. In recent years, there has been a shift from locoregional failure to distant failure for patients treated for presumed locoregional disease.[18] This in part is a reflection of subclinical distant disease present prior to the initiation of therapy. Testing for the presence of biomarkers may allow the detection of small amounts of tumor cells in a background of normal tissue. It is conceivable that the identification of a biomarker specific for head and neck tumors or of a panel of such biomarkers may allow the detection of distant microscopic disease.

[0066] As a result a non-invasive diagnostic detection of diseases, and in particular of oral cavity and oropharyngeal cancer in patients is disclosed. In particular saliva IL8

mRNA and protein are diagnostic of patients with Oral Cavity (OC) and Oropharyngeal (OP) Squamous Cell Carcinoma (SCCA), as well as IL1B, DUSP1, HA3, OAZ1, S100P and SAT also tested. This could be envisioned during routine dental visits or targeted screening of individuals at high risk of development of the disease. A home test kit can also be envisioned.

[0067] Whole saliva can be obtained from subjects through specific defined proccedures to isolate mRNA and proteins, preferably with the procedures and methods disclosed herein, Real time quantitative PCR and ELISA for the respective cytokine will be performed for IL8.

[0068] Serum IL6 mRNA and protein are also diagnostic of patients with OC and OP SCCA. this could be envisioned as a screening test for presence of occult OC and OP SCCA during routine physician's visit with blood work or targeted screening of individuals at high risk for oral cancer development. A home test kit can also be envisioned.

[0069] In particular peripheral blood can be obtained from subjects using routine clinical procedures. mRNA and proteins are isolated, preferably with an optimized procedures herein disclosed. Real time quantitative PCR and ELISA for the respective cytokine will be performed for IL6.

[0070] The method and kits can be used during routine physician's visit as part of e.g. regular physical examination. Additionally screening of high risk population for the disease can be performed. In the case of OC and OP SCCA are a) smokers and dirnkers age >45; b) African Americans; c) women age 20-40 with no associated known risk factor.

[0071] Such an assay will be have the advantage of being simple and robust. When performed on saliva will have a further advantage of using a non-invasive fluid.

[0072] The fact that no healthy control subject had a saliva or serum marker above the reported threshold is encouraging, indicating the excellent test specificity.

[0073] Analogous results were obtained for breast cancer, diabetes, and are expected for HIV.

[0074] Future refinement of the approach of the inventors may focus on several areas. More biomarkers could be added to the panel. Using oral fluid based microarray technologies, our laboratory has identified other molecular biomarkers that appear specific for OSCC, which include IL1B, DUSP1, HA3, OAZ1, S100P and SAT. A comprehensive panel of markers capturing all tumors and low-cost high-throughput technology is ideal so that early molecular detection can be applied in real-life screening. Widespread adoption of clinical innovation will depend on its cost, standardization, reproducibility, and ease of use.

[0075] The cumulative outcomes of the proposed studies will be used in a series of next steps towards the eventual creation of micro-/nano-electrical mechanical systems (MEMS/NEMS) for the ultrasensitive detection of molecular biomarkers in oral fluid. RNA and protein expression for the validated OSCC biomarkers will be selected as targets for cancer detection. The integration of these detection systems for the concurrent detection of mRNA and protein for multiple OSCC biomarkers will result in an efficient, automated, affordable system for oral fluid based cancer diagnostics.

[0076] Further details concerning reagents, conditions, compositions techniques to be used in the method and kits of the disclosure are identifiable by a person skilled in the art upon reading of the present disclosure.

[0077] The following examples are provided to describe the invention in further detail. These examples, which set forth a preferred mode presently contemplated for carrying out the invention, are intended to illustrate and not to limit the invention

Examples

Example 1: RNA Isolation and Amplification

[0078] Normal subjects

[0079] Saliva samples were obtained from ten normal donors from the Division of Otolaryngology, Head and Neck Surgery, at the Medical Center, University of California, Los Angeles (UCLA), CA, in accordance with a protocol approved by the UCLA Institutional Review Board. The following inclusion criteria were used: age 30 years; no history of malignancy, immunodeficiency, autoimmune disorders, hepatitis, HIV infection or smoking. The study population was composed of 6 males and 4 females, with an average age of 42 years (range from 32 to 55 years).

[0080] Saliva collection and processing

[0081] *Unstimulated saliva* were collected between 9am and 10am in accordance with published protocols (Navazesh, 1993). Subjects were asked to refrain from eating, drinking, smoking or oral hygiene procedures for at least one hour prior to saliva collection. Saliva samples were centrifuged at 2,600 x *g* for 15 min at 4$_o$C. Saliva supernatant was separated from the cellular phase. RNase inhibitor (Superase-In, Ambion Inc., Austin, TX, USA) and protease inhibitor (Aprotinin, Sigma, St. Louis, MO, USA) were then added into the cell-free saliva supernatant.

[0082] RNA isolation from cell-free saliva

[0083] RNA was isolated from cell-free saliva supernatant using the modified protocol from the manufacturer (QIAamp Viral RNA kit, Qiagen, Valencia, CA, USA). Saliva (560 µL), mixed 6well with AVL buffer (2,240 µL), was incubated at room temperature for 10 min. Absolute ethanol (2,240 µL) was added and the solution passed through silica columns by centrifugation at 6,000 x *g* for 1 min. The columns were then washed twice, centrifuged at 20,000 x *g* for 2 min, and eluted with 30 µL RNase free

water at 9,000 x *g* for 2 min. Aliquots of RNA were treated with RNase-free DNase (DNase I-DNA-free, Ambion Inc., Austin, TX, USA) according to the manufacturer's instructions. The quality of isolated RNA was examined by RT-PCR for three house-keeping gene transcripts: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), actin–â (ACTB) and ribosomal protein S9 (RPS9). Primers were designed using PRIMER3 software (http://www.genome.wi.mit.edu) and were synthesized commercially (Fisher Scientific, Tustin, CA, USA) as follows: 5' TCACCAGGGCTGCTTTTAACTC3' and 5'ATGACAAGCTTCCCGTTCTCAG3' for GAPDH; 5'AGGATGCAGAAGGAGATCACTG3' and 5'ATACTCCTGCTTGCTGATCCAC3' for ACTB; 5'GACCCTTCGAGAAATCTCGTCTC3' and 5'TCTCATCAAGCGTCAGCAGTTC3' for RPS9. The quantity of RNA was estimated using Ribogreen® RNA Quantitation Kit (Molecular Probes, Eugene, OR, USA).

[0084] Target cRNA preparation

[0085] Isolated RNA was subjected to linear amplification according to published method from our laboratory (Ohyama *et al.*, 2000). In brief, reverse transcription using T7-oligo-$(dT)_{24}$ as the primer was performed to synthesize the first strand cDNA. The first round of *in vitro* transcription (IVT) was carried out using T7 RNA polymerase (Ambion Inc., Austin, TX, USA). The BioArray™ High Yield RNA Transcript Labeling System (Enzo Life Sciences, Farmingdale, NY, USA) was used for the second round IVT to biotinylate the cRNA product; the labeled cRNA was purified using GeneChip® Sample Cleanup Module (Affymetrix, Santa Clara, CA, USA). The quantity and quality of cRNA were determined by spectrophotometry and gel electrophoresis. Small aliquots from each of the isolation and amplification steps were used to assess the quality by RT-PCR. The quality of the fragmented cRNA (prepared as described by Kelly, 2002) was assessed by capillary electrophoresis using the 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA).

[0086] Gene expression profiling in cell free-saliva obtained from ten normal donors, wherein on average, 60.5 ± 13.1 ng (n=10) of total RNA was obtained from 560 µL cell-free saliva samples, is reported on Table 1.

[0087] Table 1.

Subject	Gender	Age	RNA (ng)a	cRNA (~tg)'~	Present Probesc	Probe ~%"
1	F	53	60.4	44.3	3172	14.24
2	M	42	51.6	40.8	2591	11.62
3	M	55	43.2	34.8	2385	10.70
4	M	42	48.2	38.0	2701	12.12
5	M	46	60.6	42.7	3644	16.35
6	M	48	64.8	41.8	2972	13.34
7	F	40	75.0	44.3	2815	12.63
8	M	33	77.8	49.3	4159	18.66
9	F	32	48.8	41.4	2711	12.17
10	F	32	79.8	44.4	4282	19.22
Mean±SD		42±8.3	60.5±13.12	42.2±3.94	3143±665.0	14.11±2.98

[0088] The total RNA quantity is the RNA in 560p.L cell-free saliva supernatant; the eRNA quantity is after two rounds of T7 amplification. Number of probes showing present call on HG U133A microarray (detection p<0.04). Present percentage (P%) = Number of probes assigned present call / Number of total probes (22,283 for HG U133A microarray).

[0089] RT-PCR results demonstrated all 10 saliva samples contain mRNAs that encode for house keeping genes: GAPDH, ACTB and RPS9. The mRNA of these genes could be preserved without significant degradation for more than 6 months at -80oC (Fig. 1). After two rounds of T7 RNA linear amplification, the average yield of biotinylated cRNA was 42.2 ± 3.9 µg with A260/280=2.067 ***± 0.082*** (Table1). The cRNA ranged from 200 bp to 4 kb before fragmentation; and was concentrated to approximately 100bp after fragmentation. The quality of cRNA probe was confirmed by capillary electrophoresis before the hybridizations. ACTB mRNA was detectable using

PCR/RT-PCR on original sample and products from each amplification steps: first cDNA, first in vitro transcription (IVT), second cDNA and second IVT (Fig. 2).

Example 2: Microarray Profiling of Salivary mRNA

[0090] HG-U1331A Microarray analysis

[0091] The Affymetrix Human Genome U133A Array, which contains 22,215 human gene cDNA probe sets representing ~19,000 genes (*i.e.*, each gene may be represented by more than one probe sets), was applied for gene expression profiling. The array data were normalized and analyzed using Microarray Suite (MAS) software (Affymetrix). A detection *p*-value was obtained for each probe set. Any probe sets with $p < 0.04$ was assigned "present", indicating the matching gene transcript is reliably detected (Affymetrix, 2001). The total number of present probe sets on each array was obtained and the present percentage (P%) of present genes was calculated. Functional classification was performed on selected genes (present on all ten arrays, $p < 0.01$) by using the Gene Ontology Mining Tool (www.netaffx.com).

[0092] Salivary mRNA profiles of ten normal subjects were obtained using HG U133A array contains 22,283 cDNA probes. An average of 3,143 ± 665.0 probe sets ($p < 0.04$) was found on each array (n=10) with assigned present calls. These probe sets represent approximately 3,000 different mRNAs. The average present call percentage was 14.11 ± 2.98% (n=10). A reference database which includes data from the ten arrays was generated. The probe sets representing GAPDH, ACTB and RPS9 assigned present calls on all 10 arrays. There were totally 207 probe sets representing 185 genes assigned present calls on all 10 arrays with detection $p < 0.01$. These 10 genes were categorized on the basis of their known roles in biological processes and molecular functions. Biological processes and molecular functions of 185 genes in cell-free saliva from ten normal donors (data obtained by using Gene Ontology Mining Tool) are reported on table 2.

[0093] Table 2.

Biological process[a]	**Genes ,nb**	**Molecular function**[a]	**Genes,nb**
Cell growth and/or maintenance	119	Binding	118
Metabolism	93	Nucleic acid binding	89
Biosynthesis	70	RNA binding	73
Protein metabolism	76	Calcium ion binding	12
Nucleotide metabolism	10	Other binding	23
Other metabolisms	18		
Cell organization and biogenesis	2	Structural molecule	95
Homeostasis	3	Ribosomal constituent	73
Cell cycle	5	Cytoskeleton constituent	17
Cell proliferation	11	Muscle constituent	2
Transport	5		
Cell motility	8	Obsolete	15
		Transporter	4
Cell communication	34	Enzyme	20
Response to external stimulus	19	Signal transduction	10
Cell adhesion	3	Transcription regulator	7
Cell-cell signaling	5	Translation regulator	5
Signal transduction	17	Enzyme regulator	9
		Cell adhesion molecule	1
Obsolete	8		
Development	18	Molecular function unknown	6
Death	2		
Biological process unknown	11		

[0094] One gene may have multiple molecular functions or participate in different biological processes. Number of genes classified into a certain group/subgroup. The major functions of the 185 genes are related to cell growth/maintenance (119 genes), molecular binding (118 genes) and cellular structure composition (95 genes). We termed these as "Normal Salivary Core Transcriptome (NSCT)".

Example 3: Q-PCR Validation and Quantitation Analysis

[0095] Quantitative gene expression analysis by Q-PCR

[0096] Q-PCR was performed using iCyclerTM thermal Cycler (Bio-Rad, Hercules, CA, USA). A 2 µL aliquot of the isolated salivary RNA (without amplification) was

reverse transcribed into cDNA using MuLV Reverse Transcriptase (Applied Biosystems, Foster City, CA, USA). The resulting cDNA (3 µL) was used for PCR amplification using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). The primers were synthesized by Sigma-Genosys (Woodlands, TX, USA) as follows: 5' GTGCTGAATGTGGACTCAATCC3' and 5' ACCCTAAGGCAGGCAGTTG3' for interleukin 1, beta (IL1B); 5' CCTGCGAAGAGCGAAACCTG 3' and 5' TCAATACTGGACAGCACCCTCC 3' for stratifin (SFN); 5' AGCGTGCCTTTGTTCACTG 3' and 5' CACACCAACCTCCTCATAATCC 3' for tubulin, alpha, ubiquitous (K-ALPHA-1). All reactions were performed in triplicate with conditions customized for the specific PCR products. The initial amount of cDNA of a particular template was extrapolated from a standard curve using the LightCycler software 3.0 (Bio-Rad, Hercules, CA, USA). The detailed procedure for quantification by standard curve has been previously described (Ginzinger, 2002).

[0097] Real time quantitative PCR (Q-PCR) was used to validate the presence of human mRNA in saliva by quantifying selected genes from the 185 "Normal Salivary Core Transcriptome" genes. We randomly selected IL1B, SFN and K-ALPHA-1, which were assigned present calls on all 10 arrays, for validation. Q-PCR results showed that mRNA of IL1B, SFN and K-ALPHA-1 were detectable in all 10 original, unamplified, cell-free saliva. The relative amounts (in copy number) of these transcripts (n=10) are: 8.68 x 103 ± 4.15 x 103 for IL1B; 1.29 x 105 ± 1.08 x105 for SFN; and 4.71 x 106 ± 8.37 x 105 for K-ALPHA-1. The relative RNA expression levels of these genes measured by Q-PCR were similar to those measured by the microarrays (data not shown).

Example 4: mRNA Isolation from the Fluid Phase of Saliva

[0098] Patients selection

[0099] Patients were recruited from the Division of Head and Neck Surgery at the University of California, Los Angeles (UCLA) Medical Center, Los Angeles, CA; the University of Southern California (USC) Medical Center, Los Angeles, CA; and the

University of California San Francisco (UCSF) Medical Center, San Francisco, CA, over a 6 -month period. Thirty-two patients with documented primary T1 or T2 squamous cell carcinoma of the oral cavity (OC) or oropharynx (OP) were included in this study. All patients had recently been diagnosed with primary disease, and had not received any prior treatment in the form of chemotherapy, radiotherapy, surgery, or alternative remedies. An equal number of age and sex matched subjects with comparable smoking histories were selected as a control comparison group. Among the two subject groups, there were no significant differences in terms of mean age (standard deviation, SD): OSCC patients, 49.3 (7.5) years; normal subjects, 48.8 (5.7) years (Student's *t* test $P > 0.80$); gender (Student's *t* test $P > 0.90$); or smoking history (Student's *t* test $P > 0.75$). No subjects had a history of prior malignancy, immunodeficiency, autoimmune disorders, hepatitis, or HIV infection. Each of the individuals in the control group underwent a physical examination by a head and neck surgeon, to ensure that no suspicious mucosal lesion was present.

[00100] Saliva And Serum Collection And Processing

[00101] Informed consent had been given by all patients. Saliva and serum procurement procedures were approved by the institutional review board at each institution: the University of California, Los Angeles (UCLA); the University of Southern California (USC); and the University of California San Francisco (UCSF).

[00102] Saliva from 32 patients with OC or OP SCCA, and 32 unaffected age- and gender-matched control subjects were obtained for a prospective comparison of cytokine concentration.

[00103] The subjects were required to abstain from eating, drinking, smoking, or using oral hygiene products for at least one hour prior to saliva collection. Saliva collection was performed using the "draining (drooling)" method of Navazesh and Christensen,[7] for a total donation of 5 cc saliva. Saliva samples were subjected to centrifugation at 3500 rpm (2600xg) for 15 minutes at 4°C by a Sorvall RT6000D centrifuge (DuPont, Wilmington, DE). The fluid-phase was then removed, and RNAse (Superase-In, RNAse

Inhibitor, Ambion Inc., Austin, TX) and protease (Aprotinin, Sigma, St. Louis, MO; Phenylmethylsulfonylfluoride, Sigma, St. Louis, MO; Sodium Orthovanadate, Sigma, St. Louis, MO) inhibitors were then added promptly on ice. The conditions for the separation of the cellular and fluid phases of saliva were optimized to ensure no mechanical rupture of cellular elements which would contribute to the mRNA detected in the fluid phase. All samples were subsequently treated with DNAse (DNAseI-DNA-free, Ambion Inc., Austin, TX). The cell pellet was retained and stored at –80°C.

[00104] Serum from 19 patients with OC or OP SCCA, and 32 unaffected age- and gender-matched control subjects were obtained for a prospective comparison of cytokine concentration. Blood was drawn from control subjects and patients prior to treatment. Sera were collected by centrifuging whole blood at 3000 rpm (1000xg) for 10 minutes at 15°C by a Sorvall RT6000D centrifuge (DuPont, Wilmington, DE). Serum was then separated, and RNAse (Superase-In, RNAse Inhibitor, Ambion Inc., Austin, TX) and protease (Aprotinin, Sigma, St. Louis, MO; Phenylmethylsulfonylfluoride, Sigma, St. Louis, MO; Sodium Orthovanadate, Sigma, St. Louis, MO) inhibitors were then added promptly on ice. All samples were subsequently treated with DNAse (DNAseI-DNA-free, Ambion Inc., Austin, TX). The aliquots were stored at –80°C until further use.

[00105] RNA Isolation

[00106] 560 µL of saliva supernatant were then processed using the QIAamp Viral RNA mini kit (QIAGEN, Chatsworth, CA) kit. RNA was extracted according to the manufacturer's instructions. Samples were air-dried and resuspended in water treated with diethyl pyrocarbonate and were kept on ice for immediate usage or stored at –80°C. Aliquots of RNA were treated with RNAse-free DNAse (DNAseI-DNA-free, Ambion Inc., Austin, TX) according to the manufacturer's instructions. Concentrations of RNA were determined spectrophotometrically, and the integrity was checked by electrophoresis in agarose gels containing formaldehyde.

[00107] RT-PCR

[00108] RNA from each sample was reverse-transcribed in 40 µL of reaction mixture containing 2.5 U of Moloney murine leukemia virus reverse transcriptase (Applied Biosystems Inc.(ABI, Foster City, CA) and 50 pmol of random hexanucleotides (ABI, Foster City, CA) at 42°C for 45 minutes. Based on the published sequences, oligonucletide primers were synthesized commercially at Fisher Scientific (Tustin, CA) for PCR as follows: 5'AGGATGCAGAAGGAGATCACTG 3'and 5'ATACTCCTGCTTGCTGATCCAC 3' for□-actin; and 5'GAGGGTTGTGGAGAAGTTTTTG 3' and 5'CTGGCATCTTCACTGATTCTTG 3' for IL-8; and 5' CTGGCAGAAAACAACCTGAAC 3' and 5'ATGATTTTCACCAGGCAAGTC 3' for IL-6. Amplification of the complementary DNA (cDNA) was carried out using 50 cycles at 95°C for 20 seconds, 60°C for 30 seconds, and 72°C for 30 seconds; followed by a final extension cycle of 72°C for 7 minutes. Specificity of the PCR products was verified by the predicted size and by restriction digestion. To establish the specificity of the responses, negative controls were used in which input RNA was omitted or in which RNA was used but reverse transcriptase omitted. As a positive control, mRNA was extracted from total salivary gland RNA (Human Salivary Gland Total RNA, Clontech, Palo Alto, CA). To ensure RNA quality, all preparations were subjected to analysis of expression.

[00109]

[00110] To our knowledge, there have been no reports about the isolation of messenger RNA (mRNA) from the fluid phase of saliva. By using reverse transcriptase-polymerase chain reaction (RT-PCR), we tested the hypothesis that IL-6 and IL-8 mRNA transcripts are present in the fluid phase in saliva. The RT-PCR studies showed that saliva and serum contained mRNA encoding for IL-6 and IL-8. The PCR products had the sizes (95 bp and 88 bp, respectively) that were expected from the selected primers (**Figure 3).** The same-sized products were expressed in the positive control.

[00111] In order to ensure that the RNA and protein analyzed were from the fluid phase of saliva only (and to ensure the lack of contamination by intracellular

components), we optimized the centrifugation speed for the saliva and serum samples. We utilized genomic DNA as a marker of cell lysis and spillage of intracellular components. We performed PCR for the housekeeping genes □-actin and ubiquitin on whole saliva and serum samples, and samples that had been centrifuged at various speeds. We were able to determine the optimal centrifugation speeds at which there was no spillage of intracellular contents (**Figure 4**).

Example 5: Elevated IL-8 Cytokine Levels in Saliva from Patients with OSCC

Real Time PCR for Quantification of IL-6 and IL-8 mRNA Concentrations in Saliva and Serum from Patients and Normal Subjects

[00112] To analyze quantitatively the result of RT-PCR, we used quantitative real-time PCR (Bio-Rad iCycler, Thermal Cycler, Bio-Rad Laboratories, Hercules, CA). Each sample was tested in triplicate. The amplification reactions were carried out in a 20 µL mixture, using iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA). After initial denaturation at 95°C for 3 minutes, 50 PCR cycles were performed at 60°C for 20 seconds, then 20 seconds at 72°C, then 20 seconds at 83°C, followed by 1 minute at 95°C, then followed by a final 1 minute extension at 55°C. Aliquots were taken from each well and checked by electrophoresis in agarose gels in order to ensure the specificity of the products.

ELISA for Quantification of IL-6 and IL-8 Protein Concentrations in Saliva and Serum from Patients and Normal Subjects

[00113] ELISA kits for IL-6 and IL-8 were used (Pierce Endogen, Rockford, IL) according to the manufacturer's protocol. Each sample was tested in duplicate in each of two replicate experiments. After development of the colorimetric reaction, the absorbance at 450 nm was quantitated by an eight channel spectrophotometer (EL800 Universal Microplate Reader, BIO-TEK Instruments Inc., Winooski, VT), and the absorbance readings were converted to pg/ml based upon standard curves obtained with recombinant cytokine in each assay. If the absorbance readings exceeded the

linear range of the standard curves, ELISA assay was repeated after serial dilution of the supernatants. Each sample was tested in at least two ELISA experiments, and the data were calculated from the mean of tests for each sample.

[00114]

[00115] On demonstrating that IL-6 and IL-8 mRNA transcripts were present in the fluid phase in saliva, we prospectively examined and compared the levels of IL-6 and IL-8 in the saliva of unaffected subjects and patients with OSCC using quantitative real time PCR (qRT-PCR) and ELISA. Saliva from 32 patients with OSCC, and 32 age- and gender-matched control subjects were obtained. Among the subject groups, there were no significant differences in terms of age, gender, alcohol consumption, or smoking history ($P > 0.75$). **Figure 5 A, B** shows that IL-8 at both the mRNA and protein levels, was detected in higher concentrations in the saliva of patients with OSCC when compared with control subjects (*t* test, $P < 0.01$). There was a significant difference in the amount of IL-8 mRNA expression between saliva from OSCC patients and disease-free controls. The mean copy number was 1.1×10^8 for the OSCC group, and 2.6×10^6 for the control group. The difference between the two groups was highly statistically significant ($P<0.0008$).

[00116] Our ELISA findings are illustrated in **Figure 5B**. The levels of IL-8 in the saliva of OSCC patients were significantly higher (720 pg/dL) than those in the saliva of the control group (250 pg/dL) ($P<0.0001$). To ensure that the elevated levels of IL-8 protein in saliva were not due to an elevation of total protein levels in the saliva of OSCC patients, we compared the total protein concentrations in saliva among the two groups. No significant differences were found ($P > 0.05$). When we compared the IL-6 levels between the two groups, we did not find significant differences in the salivary concentration at either the mRNA or the protein level. Within the sample size studies, we were unable to detect differences between smoking and nonsmoking subjects (data not shown).

[00117] Example 6: Elevated IL-6 Cytokine Levels in Serum from Patients with OSCC

[00118] We also examined and compared the levels of IL-6 and IL-8 in the serum of unaffected subjects and patients with OSCC using qRT- PCR and ELISA. Serum from 19 patients with OSCC, and 32 age- and gender-matched control subjects were prospectively obtained. Among the subject groups, there were no significant differences in terms of age, gender, alcohol consumption, or smoking history ($P > 0.75$). **Figure 6A, B** shows that IL-6 at both the mRNA and protein levels, was detected in higher concentrations in the serum of patients with OSCC when compared with control subjects (*t* test, $P < 0.001$). We noted a significant difference in the amount of IL-6 mRNA expression between serum from OSCC patients and disease-free controls. The mean copy number was 5.2×10^4 for the OSCC group, and 3.3×10^3 for the control group.

[00119] The difference between the two groups was highly statistically significant ($P<0.0004$). Our ELISA findings are illustrated in **Figure 6B**. The mean levels of IL-6 in the serum of OSCC patients were significantly higher (87 pg/dL) than those in the serum of the control group (0 pg/dL) ($P<0.0001$). When we compared the IL-8 levels between the two groups, we did not find significant differences in the serum concentration at either the mRNA or the protein level. Within the sample size studies, we were unable to detect differences between smoking and nonsmoking subjects (data not shown).

Example 7: ROC and Sensitivity/specificity Analysis

Statistical analysis of our data demonstrates the specificity and sensitivity of these biomarkers for HNSCC, and their predictive value.

Statistical Analysis

[00120] The distributions of patient demographics were calculated overall and separately for OSCC cases and controls, and were compared between the two arms with either the Student's *t*-test for continuous measures or two-by-two Chi-square tables for categorical measures. The distributions of IL-6 and IL-8 levels in saliva and serum were computed and compared between the OSCC cases and controls using two independent group *t*-tests. Differences were considered significant for *P* values less than 0.01. Due to the range of the IL-6 and IL-8 levels, log transformations of these measures were also used in the analyses. Data were expressed as the mean ± SD. Age, gender, and smoking history were controlled at the group level in the experimental design; these patient factors were also adjusted in the analyses when comparing IL-6 and IL-8 through regression modeling.

[00121] Using the binary outcome of the disease (OSCC cases) and non-disease (controls) as dependent variables, logistic regression models were fitted to estimate the probability of developing OSCC as a function of each of the potential biomarkers (IL-6 or IL-8), controlling for patient age, gender, and smoking history. Using the fitted logistic models, receiver operating characteristic (ROC) curve analyses were conducted to evaluate the predictive power of each of the biomarkers[8][9][10]. Through the ROC analyses, we calculated sensitivities and specificities by varying the criterion of positivity from the least (cut at probability of 0) to the most stringent (cut at probability of 1). The optimal sensitivity and specificity was determined for each of the biomarkers, and the corresponding cutoff/threshold value of each of the biomarkers was identified. The biomarker that has the largest area under the ROC curve was identified as having the strongest predictive power for detecting OSCC.

Clinical Data

[00122] The mean (SD) age of the patients with OSCC was 49.3 (7.5) years (range, 42-67 years) vs. 48.8 (5.7) years (range, 40-65 years) in the control group; (Student's *t* test $P > 0.80$). Among the two subject groups, there were no significant differences in terms of age (mean age): OSCC patients, 49.3 years; normal subjects, 48.8 years

(Student's *t* test $P > 0.80$); gender (Student's *t* test $P > 0.90$); or smoking history (Student's *t* test $P > 0.75$).

[00123]

[00124] ROC (Receiver Operating Characteristic) curves, plots of sensitivities versus 1-specificities, were generated for each of the potential biomarkers. Age, gender, and smoking history were controlled as described above. The areas under the ROC curves were calculated, as measures of the utility of each biomarker for detecting OSCC.

[00125] The calculated ROC values (for predicting OSCC) were 0.978 for IL-8 in saliva; and 0.824 for IL-6 in serum. Based on the distribution of sensitivities and specificities, thresholds of biomarkers were chosen for detecting OSCC. Based upon our data, for IL-8 in saliva, a threshold value of 600 pg/dL yields a sensitivity of 86% and a specificity of 97%.

[00126] Similarly, for IL-6 in serum, a threshold value of greater than 0 pg/dL yields a sensitivity of 64% and a specificity of 81%. Figure 7A and Figure 7B are the ROC curves for IL-8 in saliva and IL-6 in serum, respectively. The detailed statistics of the area under the ROC curves, the threshold values, and the corresponding sensitivities and specificities for each of the potential biomarkers in saliva and in serum are listed in Table 3.

[00127] The detailed statistics of the area under the ROC curves, the threshold values, and the corresponding sensitivities and specificities for each of the potential biomarkers in saliva and in serum are listed in table 3 below.

Table 3:

Biomarker	Area under ROC	Threshold/Cutoff	Sensitivity	Specificity
IL-8 saliva protein	0.978	600 pg/mL	86%	97%
IL-6 serum protein	0.824	> 0 pg/mL	57%	100%

IL-8 saliva protein & IL-6 serum protein	0.994	> 600 pg/ml > 0 p/ml	99%	90%

[00128] The disclosures of each and every publication and reference cited herein are hereby incorporated herein by reference in their entirety.

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Claims

1. A method to isolate mRNA from saliva comprising:

providing a cell-free saliva supernatant; and

isolating mRNA from the cell free saliva supernatant.

2. The method of claim 1, wherein providing a cell-free saliva supernatant comprises:

providing unstimulated saliva; and

deriving the cell-free saliva supernatant from the unstimulated saliva.

3. A method to perform transcriptome analysis of saliva comprising

providing cell-free saliva supernatant; and

detecting a transcriptome pattern in the saliva supernatant.

4. The method of claim 3, wherein detecting a transcriptome pattern in the saliva supernatant is performed by microarray assay.

5. The method of claim 4, wherein detecting a transcriptome pattern in the saliva supernatant is performed by high-density oligonucleotide microarray assay.

6. The method of claim 3, wherein detecting a transcriptome pattern in the saliva supernatant is performed by quantitative PCR analysis or RT-PCR analysis.

7. A method to detect genetic alterations in an organ by analyzing a bodily fluid draining from the organ, the bodily fluid being saliva, the method comprising:

providing cell-free saliva supernatant;

detecting a transcriptome pattern in the saliva supernatant; and

comparing the transcriptome pattern with a predetermined pattern, the predetermined pattern being indicative of a common pattern transcriptome of normal cell-free saliva.

8. A method to detect genetic alteration of a gene in an organ by analyzing a bodily fluid draining from the organ, the bodily fluid being saliva, the method comprising:

providing cell-free saliva supernatant;

detecting an mRNA profile of the gene in the saliva supernatant; and

comparing the mRNA profile of the gene with a predetermined mRNA profile of the gene, the predetermined mRNA profile of the gene being indicative of the mRNA profile of the gene in normal cell-free saliva.

9. A method to diagnose an oral or systemic disease in a subject, the method comprising:

providing cell-free saliva supernatant of the subject;

detecting in the cell-free saliva supernatant an mRNA profile of a gene associated with the disease; and

comparing the RNA profile of the gene with a predetermined mRNA profile of the gene, the predetermined mRNA profile of the gene being indicative of the presence of the disease in the subject.

10. The method of claim 9, wherein the disease is a cancer of the oral cavity and/or of oropharynx and the gene is selected from the group consisting of the gene coding for IL8, IL1B, DUSP1, HA3, OAZ1, S100P and SAT.

11. The method of claim 9 or 10, wherein the disease is a cancer of the oral cavity and/or oropharynx and the gene is the gene coding for IL8.

12. The method of any one of claims 9 to 11, wherein the disease is oropharyngeal Squamous cell carcinoma, head and neck squamous cell carcinoma and/or breast cancer.

13. The method of any one of claims 9 to 11, wherein the disease is diabetes.

14. A method to diagnose an oral or systemic disease in a subject, the method comprising:

providing cell-free saliva supernatant of the subject;

detecting in the cell-free saliva supernatant a transcriptome pattern associated with the disease; and

comparing the transcriptome pattern with a predetermined pattern,

recognition in the transcriptome pattern of characteristics of the predetermined pattern being diagnostic for the disease in the subject.

15. The method of claim 14, wherein the disease is a cancer of the oral cavity and/or of oropharynx and transcriptome includes transcripts is selected from the group consisting of transcripts for IL8, IL1B, DUSP1, HA3, OAZ1, S100P and SAT.

16. The method of claim 14, wherein disease is oropharyngeal Squamous cell carcinoma or head and neck squamous cell carcinoma.

17. The method of claim 14, wherein the disease is oropharyngeal Squamous cell carcinoma, head and neck squamous cell carcinoma or breast cancer.

18. The method of claim 14, wherein the disease is diabetes.

19. A method for diagnosing a cancer in a subject, the method comprising:

providing a bodily fluid of the subject;

detecting in the bodily fluid a profile of a biomarker, the biomarker selected from the group consisting of IL6, IL8 IL1B, DUSP1, HA3, OAZ1, S100P and SAT,

comparing the profile of the biomarker with a predetermined profile of the biomarker,

recognition in the profile of the biomarker of characteristics of the predetermined profile of the biomarker being diagnostic for the cancer.

20. The method of claim 19, wherein the biomarker is selected from the group consisting of IL8, IL1B, DUSP1, HA3, OAZ1, S100P and SAT, the bodily fluid is saliva and detecting a profile of a biomarker is performed by detecting the mRNA profile of the biomarker.

21. The method of claim 19, wherein the biomarker is IL6, the bodily fluid is blood serum and detecting a profile of a biomarker is performed by detecting the mRNA profile of the biomarker.

22. The method of claim 19, wherein the biomarker is IL6, the bodily fluid is blood serum and detecting a profile of a biomarker is performed by detecting the protein profile of the biomarker.

23. The method of claim 19, wherein the disease is diabetes breast cancer oropharyngeal Squamous cell carcinoma and/or head and neck squamous cell carcinoma.

24. A kit for the diagnosis of an oral and/or systemic disease, the kit comprising:

a identifyer of a biomarker in a bodily fluid, the biomarker selected from the group consisting of IL8, IL1B, DUSP1, HA3, OAZ1, S100P and SAT; and

a detector for the identifier,

the identifier and the detector to be used in detecting the bodily fluid profile of the biomarker of the method of any one of claims 14 to 17 or 18 to 21 , wherein

the identifier is associated to the biomarker in the bodily fluid, and the detector is used to detect the identifier, the identifier and the detector thereby enabling the detection of the bodily fluid profile of the biomarker.

25. The method of claim 24, wherein the disease is oral cavity and oropharyngeal squamous cell carcinoma.

26. The method of claim 24, wherein the disease is head and neck squamous cell carcinoma.

27. The method of claim 24, wherein the disease is breast cancer.

28. The method of claim 24, wherein the disease is diabetes.

29. The method of claim 24, wherein the disease is HIV.

30. A method comprising:

using salivary mRNAs as biomarkers for oral and/or systemic diseases

31. The method of claim 30, wherein the mRNA codifies for IL8, IL1B, DUSP1, HA3, OAZ1, S100P and SAT.

32. The method of claim 23, wherein the disease is oral cavity and oropharyngeal squamous cell carcinoma.

33. The method of claim 23, wherein the disease is head and neck squamous cell carcinoma.

34. Methods and kits as herein disclosed claimed and exemplified.

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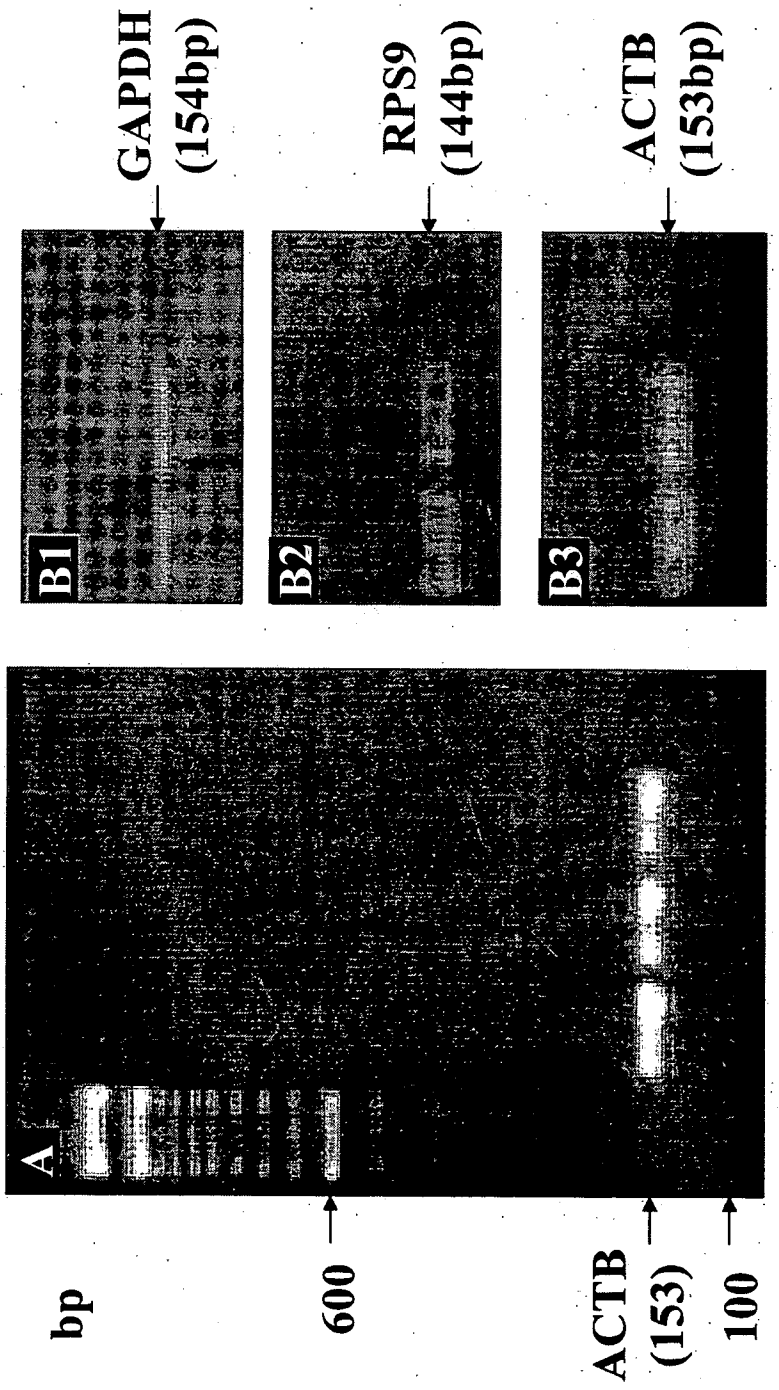
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RPS9
(144bp)
ACTB
(153bp)
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B2
B3
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A
bp
600
ACTB
(153)
100

Figure 1

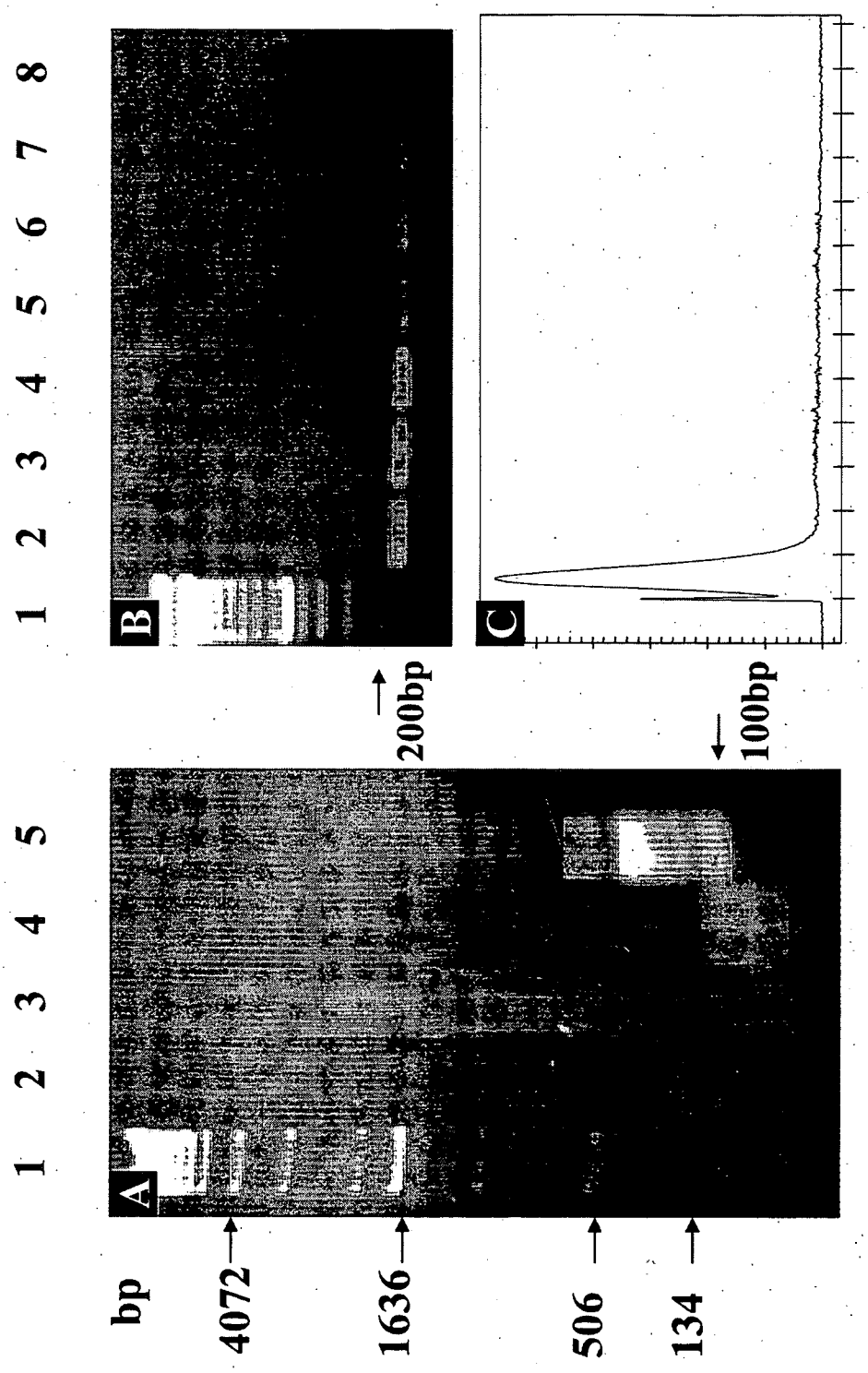
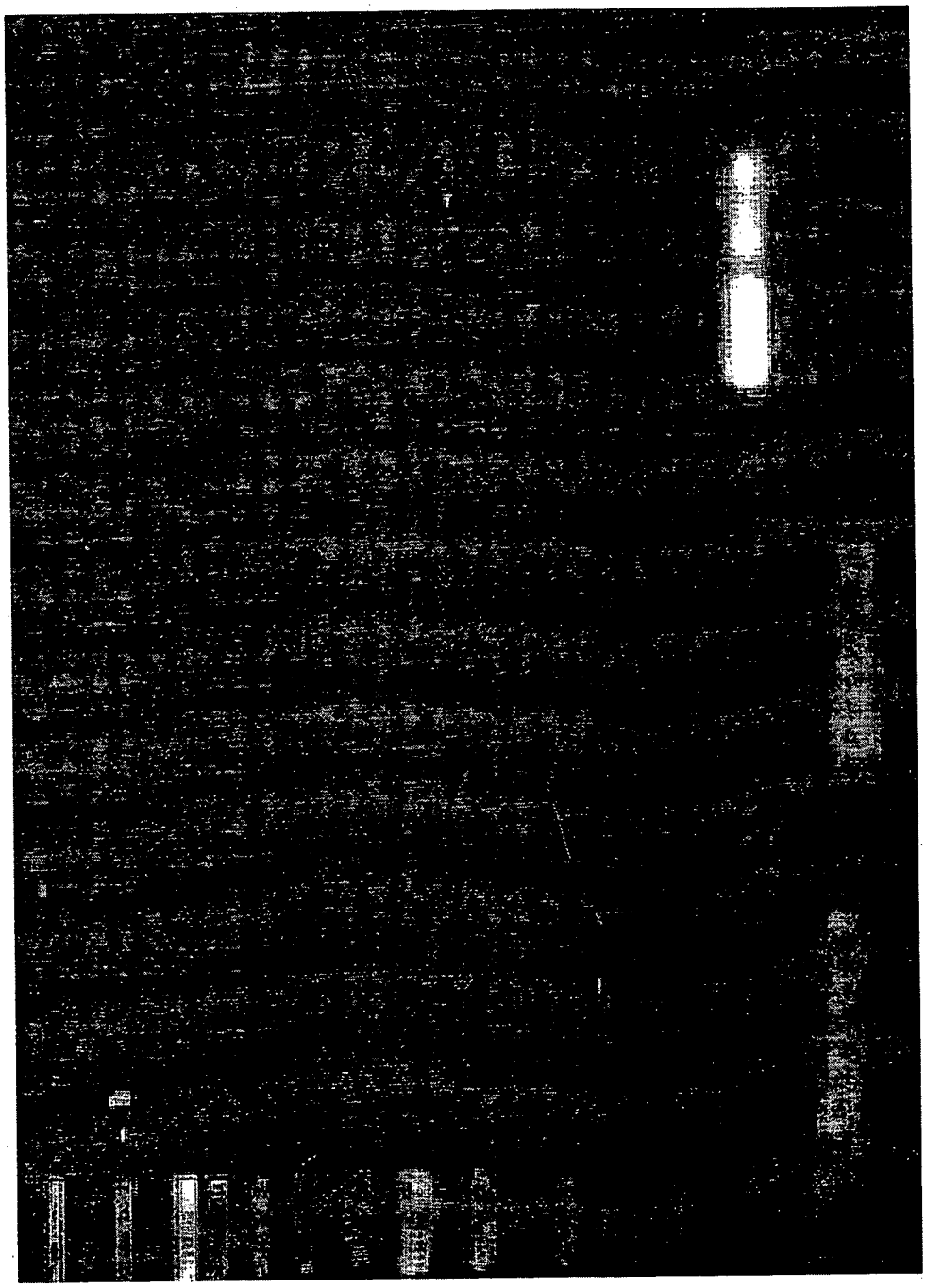


Figure 2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Ctrl (+)
Ctrl (-)
Saliva
Ctrl (+)
Ctrl (-)
Saliva
Ctrl (+)
IL-6
IL-8
β-Actin

Figure 3

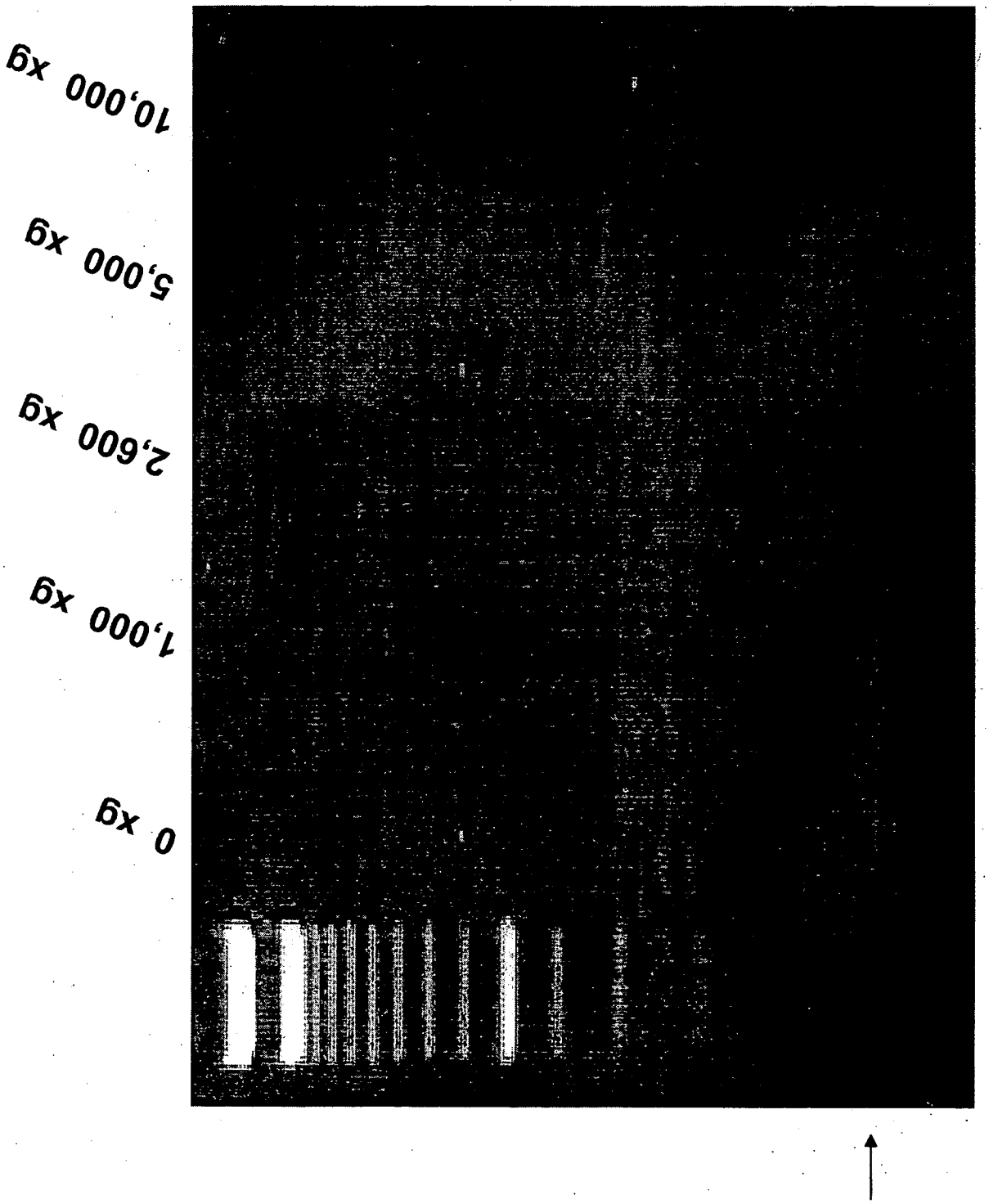
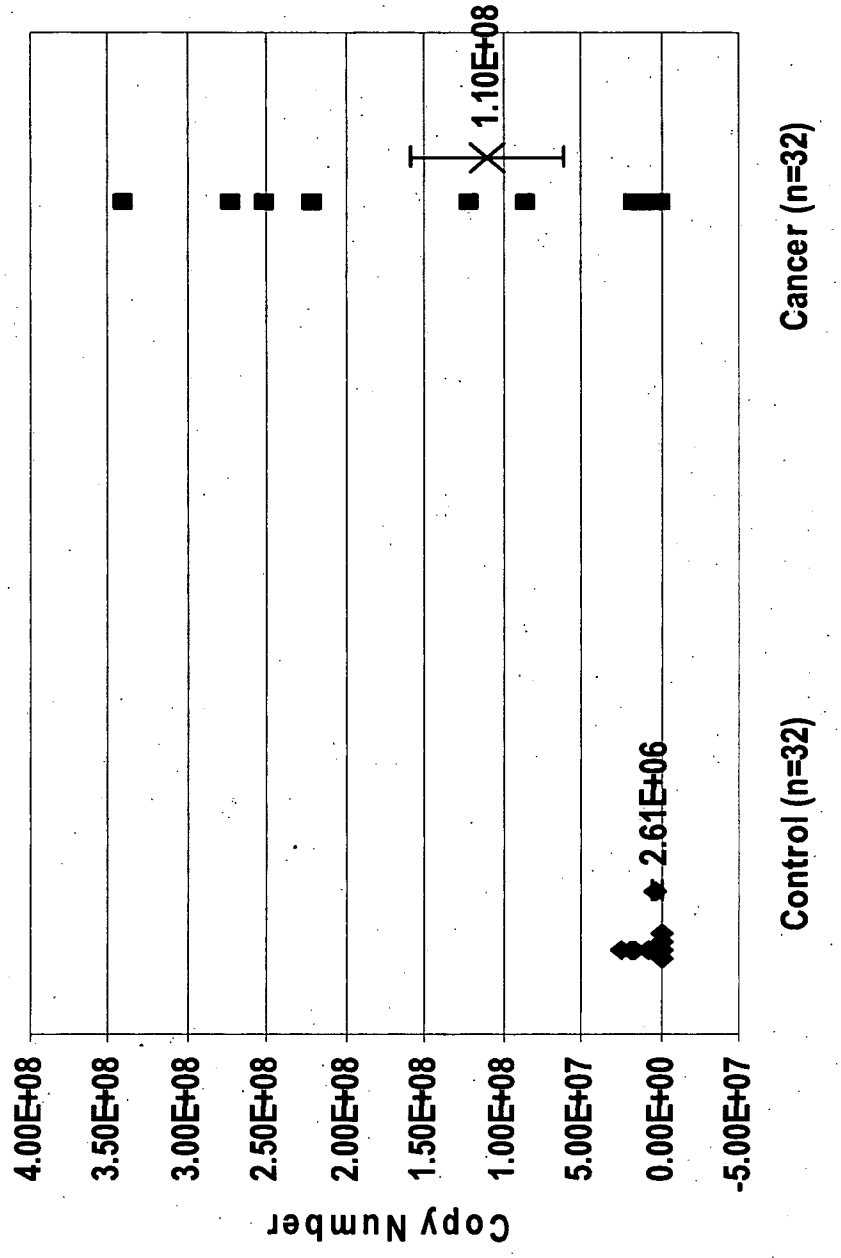


Figure 4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1.10E+08
2.61E+06
Cancer (n=32)
Control (n=32)

Figure 5A

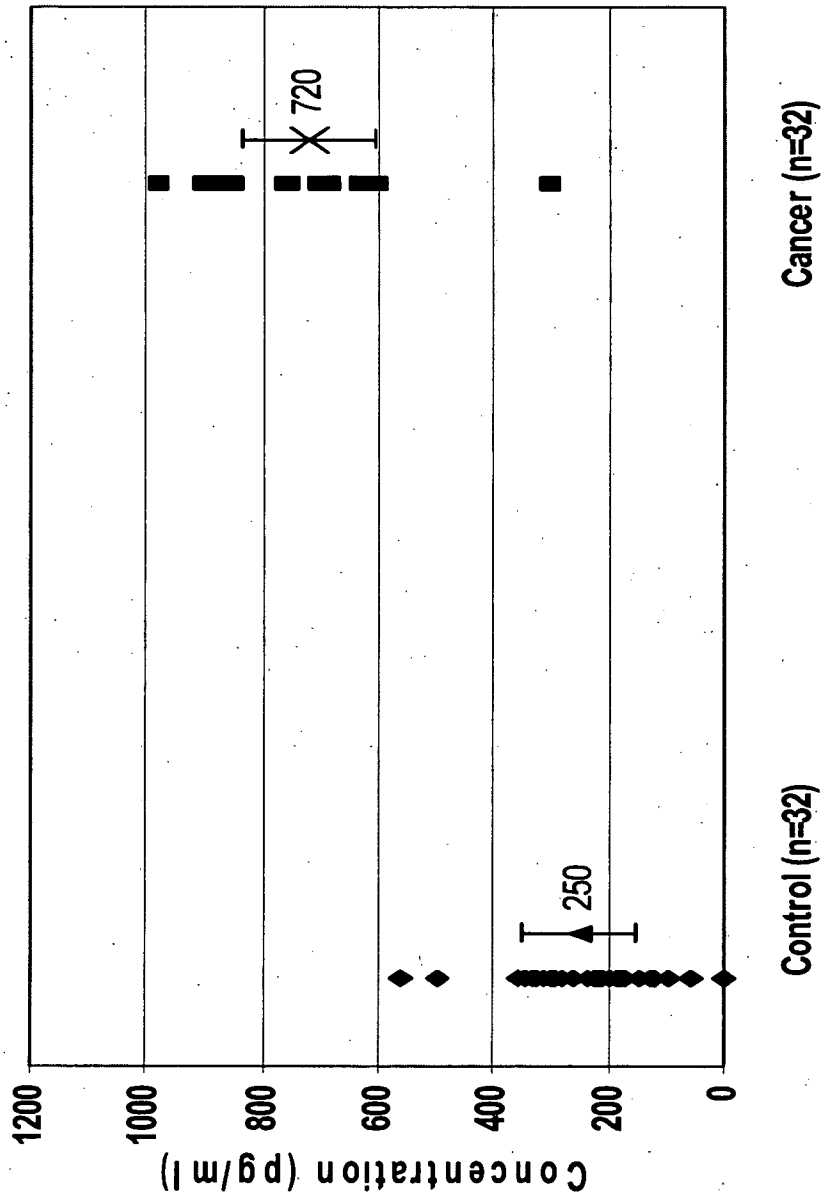
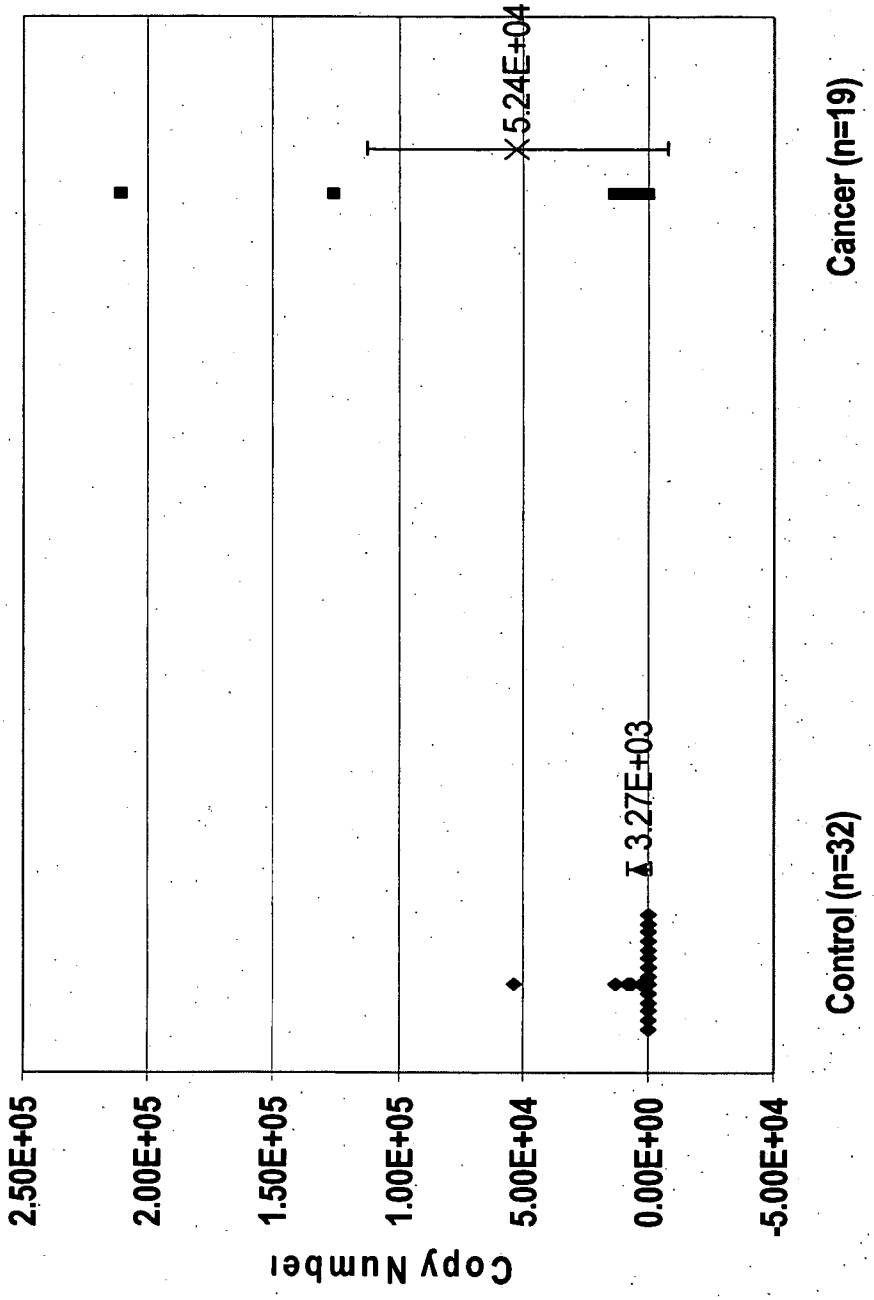

720
250
Cancer (n=32)
Control (n=32)
1200
1000
800
600
400
200
0
Concentration (pg/ml)

Figure 5/3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3.27E+03
Cancer (n=19)
Control (n=32)

Figure 6A

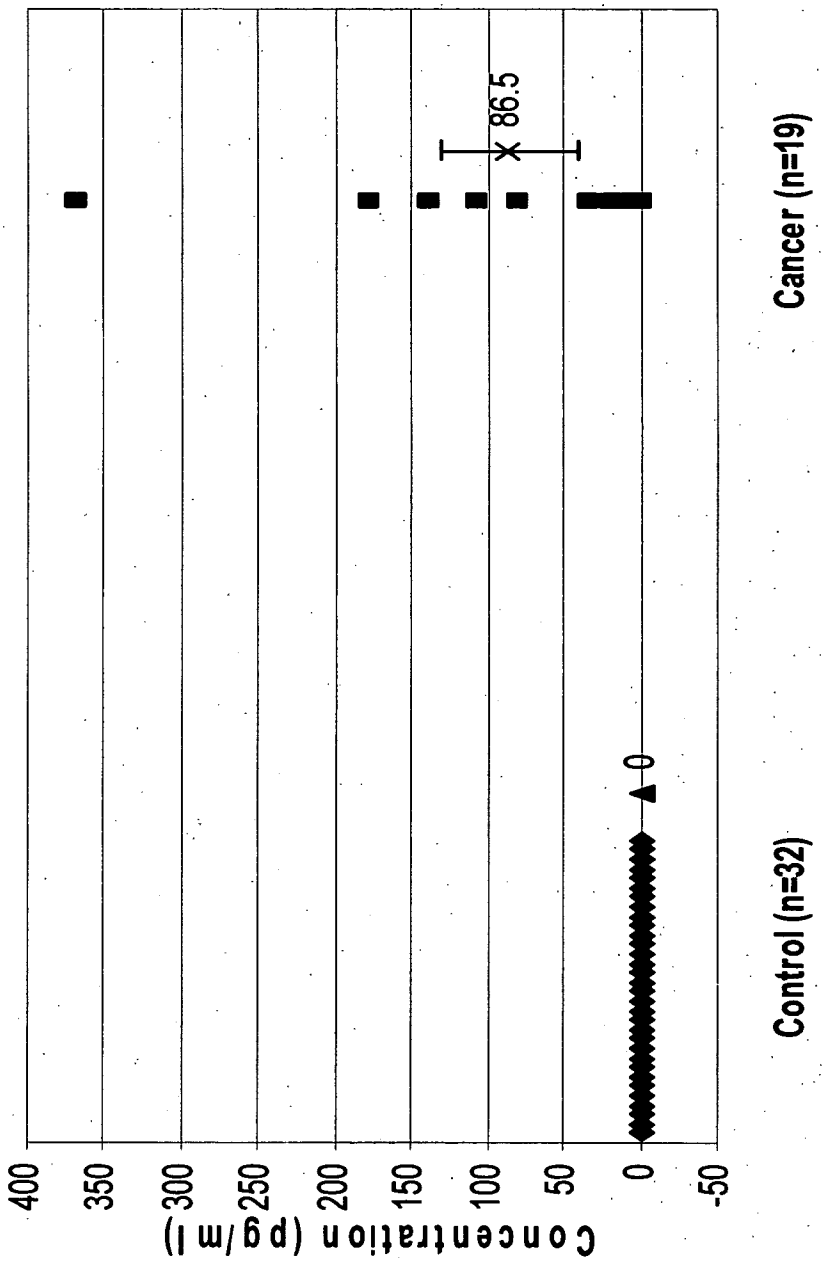
Concentration (pg/ml)
400
350
300
250
200
150
100
50
0
-50
86.5
0
Cancer (n=19)
Control (n=32)

Figure 6B

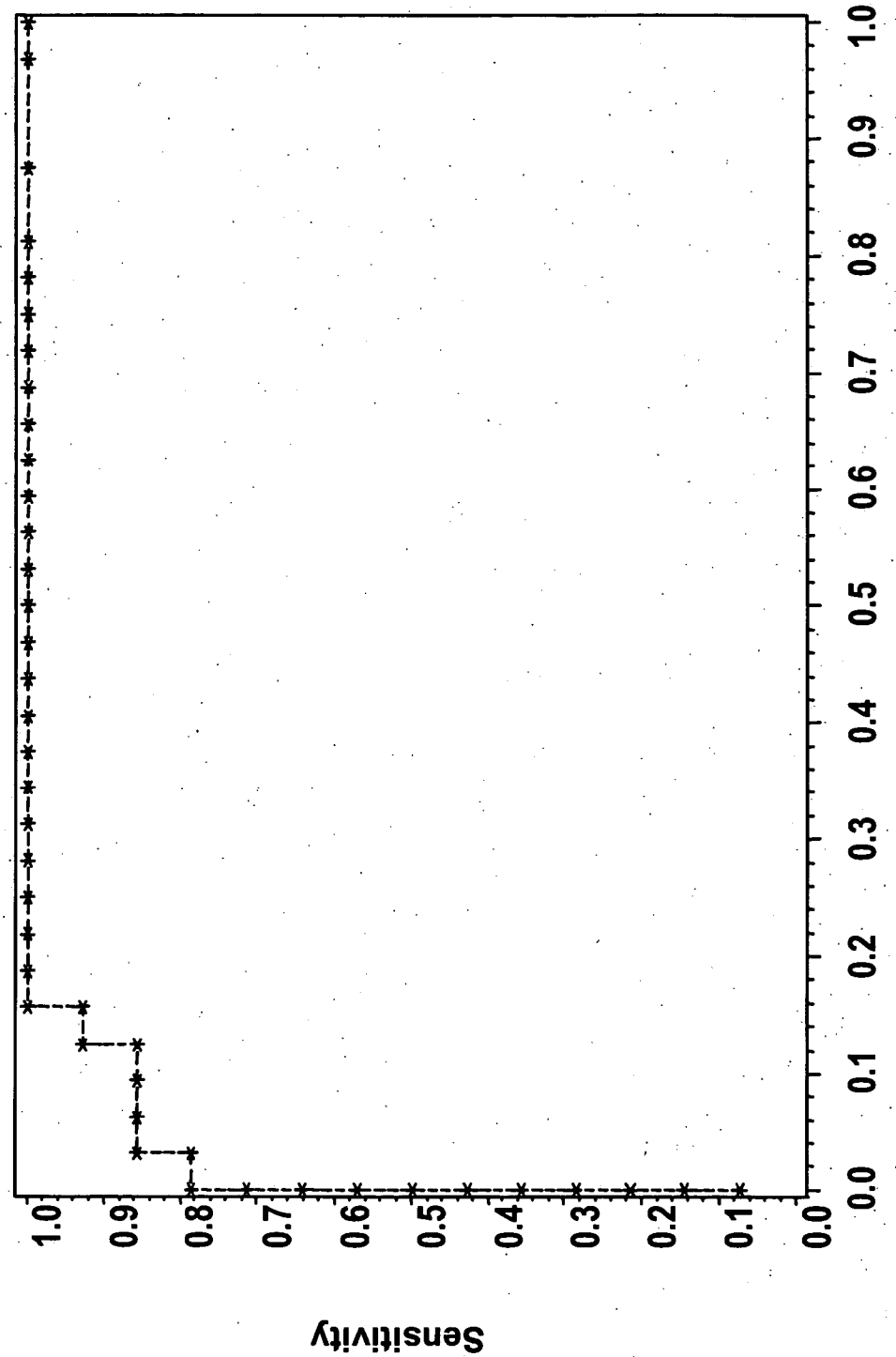
Sensitivity
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
1-Specificity
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0

Figure 7A

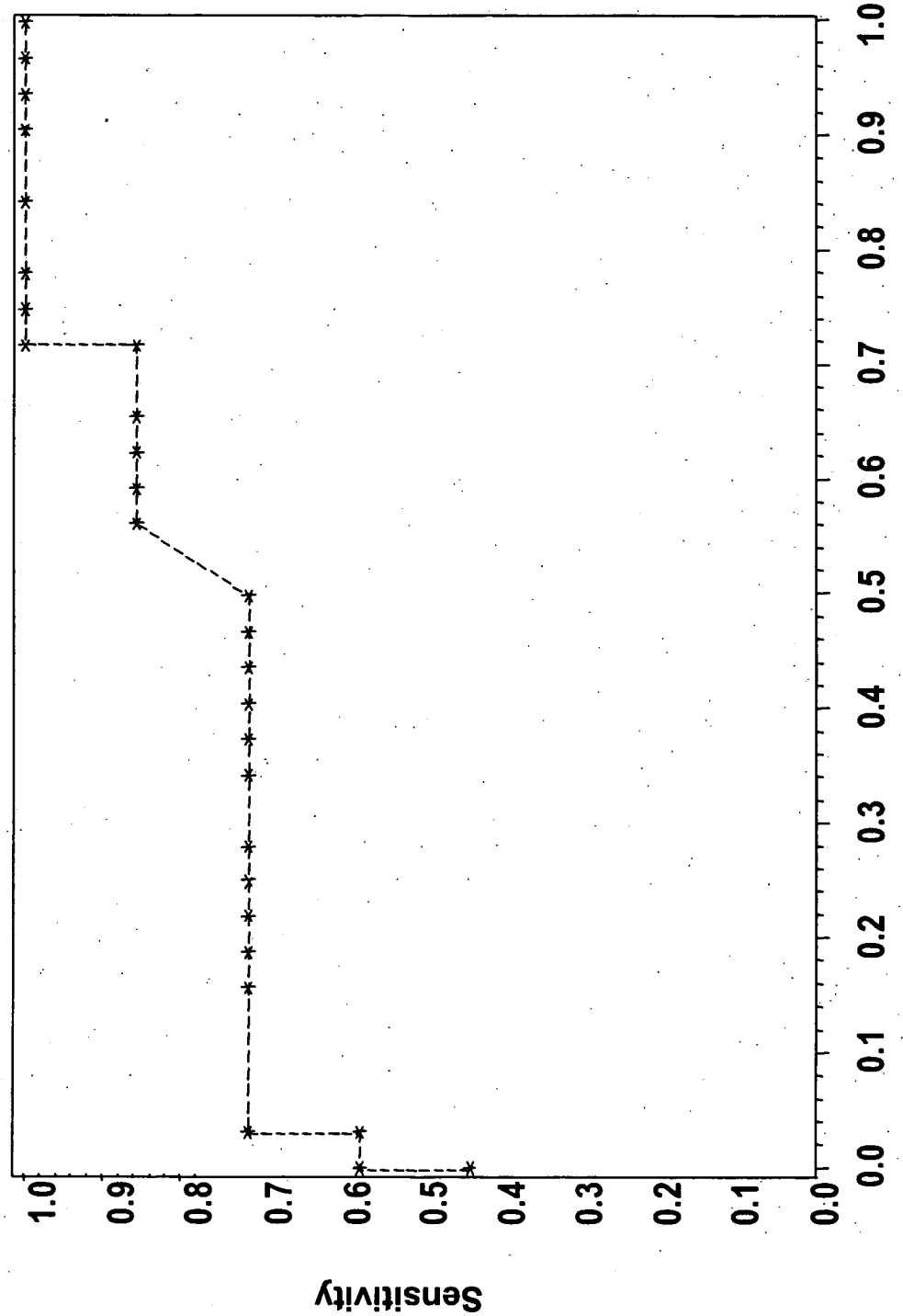
Sensitivity
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
1-Specificity
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0

Figure 7B